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Alternating patterns of cell surface properties and neural crest cell migration during segmentation of the chick hindbrain

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Summary

The developing chick hindbrain is transiently divided into a series of repeating units or rhombomeres. Recent work has shown that an alternating periodicity exists both in the cell surface properties of rhombomeres and in the segmental origin of hindbrain neural crest cells. Experiments in which rhombomeres from different axial levels were confronted in the absence of an inter-rhombomere boundary showed that odd-numbered segments 3 and 5 combined without generating a boundary, as did even-numbered segments 2, 4 and 6. When rhombomeres originating from adjacent positions, or three rhombomeres distant from one another were combined, a new boundary was regenerated.

Mapping of the migration pathways of neural crest cells showed that odd-numbered and even-numbered rhombomeres share properties with respect to the production of neural crest cells. In the hindbrain region the neural crest is segregated into streams. Neural crest cells migrating from rhombomeres 1 and 2, rhombomere 4 and rhombomere 6 respectively populate distinct cranial nerve ganglia and branchial arches. In contrast, rhombomeres 3 and 5 are free of neural crest cells.

Key words: rhombomere, hindbrain, neural crest, branchial arch, chick.

Introduction

Segmentation is a widely employed strategy in development. In this manner a uniform field may be subdivided into several territories which become autonomous and pursue different developmental fates. This process allows the generation of similar classes of cells in adjacent territories, but with distinct positional identities, and in the case of neurons, distinct axonal trajectories. In the vertebrate head, the most obvious manifestations of segmentation are the branchial arches, and the segmental organisation of the hindbrain. During an early phase of development, the hindbrain is divided into repeating units or rhombomeres, with intervening rhombomere boundaries (Lumsden and Keynes, 1989). Pairs of rhombomeres lie in register with individual branchial arches. Neural crest cells, originating from the dorsal neuroepithelium of the hindbrain, populate the sensory ganglia and the subjacent branchial region, so matching positional values between the neuroepithelium and the outlying tissues.

Segmentation is first evident in the chick embryo hindbrain between stages 9 and 12 (Hamburger and Hamilton, 1951), when rhombomere boundaries appear in a stereotyped sequence (Vaage, 1969). Single-cell marking experiments have shown that from the earliest morphological appearance of rhombomeres, cell mixing is restricted at their boundaries (Fraser *et al.*

1990). When a single cell was injected with a fluorescent tracer dye and the embryo grown up over 48 h, the resulting clone of cells mixed extensively with unlabelled, clonally-unrelated cells. Despite this capacity for cell mixing, the progeny of cells labelled after the appearance of adjacent boundaries were nevertheless confined to single rhombomeres. Thus, rhombomeres are units of cell lineage restriction in the same way as compartments in the imaginal discs of insects (Garcia-Bellido *et al.* 1973). Rhombomere boundaries might be expected to play some role in promoting this compartmentalisation by preventing cell movement between adjacent rhombomeres.

As development progresses, rhombomere boundaries become increasingly specialised in their cytoarchitecture and molecular environment. Some of these factors may be related to the maintenance of separate, non-mixing cell populations. One early indicator of this is the unusual distribution of S-phase nuclei in boundaries compared with inter-boundary regions. S-phase nuclei visualised by incorporation of bromodeoxyuridine were located in a tight band close to the basal surface of the neuroepithelium. In the boundaries, however, many S-phase nuclei occupied a more apical position, suggesting that the normal apical–basal movement of nuclei was reduced or absent in these regions (Guthrie *et al.* 1991). The tendency for nuclei to collect near the apical surface leaves large intercellular spaces basally that may provide channels for trans-

versely growing axons. By stage 17, boundaries display a distinctive morphology, with the neuroepithelial cells fanning out towards the ventricular surface, and constricted at their basal ends, while cells within interboundaries have the complementary morphology (Guthrie *et al.* 1991). The extracellular matrix molecule laminin, Ng-CAM and the low-polysialic acid form of N-CAM are prevalent within boundary regions (Lumsden and Keynes, 1989), and boundaries display an increased tendency to bind peanut agglutinin (Layer and Alber, 1990).

The onset of neurogenesis in the hindbrain occurs soon after boundaries are established, at stage 12. Conforming to the segmental pattern, neuronal differentiation in even-numbered rhombomeres precedes that in odd-numbered rhombomeres (Lumsden and Keynes, 1989). Branchiomotor nerve nuclei are also arranged segmentally. Neurons of the trigeminal nucleus (V) occupy rhombomeres 2 and 3 with their exit point within r2, while those of the facial (VII) nucleus occupy r4 and r5, exiting in r4. Each pair of segments supplies the motor outflow for a single branchial arch, with nerves V, VII and IX corresponding to arches 1, 2 and 3 respectively (Lumsden and Keynes, 1989). Cranial sensory ganglia lie adjacent to the even-numbered rhombomeres; their central axons entering the brain through the common conduit used by the outgoing motor axons.

One prominent feature of hindbrain development is thus the way in which adjacent domains, which are lineage compartments, eventually develop distinct phenotypes. Groups of neuroepithelial cells are separated early on by boundary regions containing a defined molecular environment. The conformation of the branchiomotor nuclei shows, however, that growing axons can cross boundaries, since the axons of neurons in odd-numbered segments must cross a boundary to reach their exit point. The significance of boundaries as barriers to cell movement may, therefore, be more important with respect to neuroepithelial cell behaviour early in development. Later on, the dominant theme may be the pairwise development that matches patterning within the neuroepithelium with that in the outlying branchial arches. In this review we will describe the way in which cell surface properties within the hindbrain and the deployment of the neural crest both conform to this paired pattern of development.

Alternation of cell surface properties

The observation of lineage restriction at rhombomere boundaries raises the question of how this is accomplished. Clonal restriction is first evident at a time preceding the manifestation of the most obvious boundary characteristics. Furthermore, the conspicuousness of boundaries in the hindbrain may be incidental. Indeed, there have been suggestions that other, less prominent lineage boundaries exist elsewhere in the nervous system (Lim *et al.* 1991). The possibility arises therefore, that it is not so much the

boundaries themselves that are important in preventing cell mixing, but some more cryptic properties of the cells that lie on either side of them. In *Drosophila*, for example, some lineage compartment boundaries are morphologically visible, while others, such as the anterior-posterior boundary in the wing, are not (Garcia-Bellido *et al.* 1973).

Our strategy for examining the role of boundaries in separating adjacent rhombomeres was to look at the conditions necessary for the reestablishment of a boundary. Specifically, the question asked was whether, from the earliest stages, the boundary cells constituted a special population, in whose absence cells from adjacent rhombomeres could mix freely, or whether, in the absence of a boundary, cells from adjacent rhombomeres could interact to recreate a new boundary. Experiments that suggested the latter possibility were those in which a 20 μm wide strip of boundary cells were removed from the hindbrain of a stage 10–11 embryo, by aspiration with a micropipette. Within 2–4 h the continuity of the neuroepithelium had been restored, and within 24–36 h boundary properties, such as the normal contour and a collection of neurofilament-positive axons beneath the boundary ridge, had been regenerated (Guthrie and Lumsden, 1991). This suggests that in the absence of boundary cells, the apposition of cells on either side triggers the regeneration of the missing tissue. Presumably cells from different levels can recognise surface differences, leading either to cell proliferation, or immigration of cells to fill the gap.

Is boundary regeneration a phenomenon that occurs only when cells from adjacent levels confront one another? Or does a similar boundary regeneration take place if cells from various different axial levels in the hindbrain are confronted in the absence of a boundary? In order to address these questions donor to host grafting experiments were used. The intention was not to test the developmental commitment of the piece of grafted tissue, but to confront two segments of known identity and fate, and ask whether a new boundary would be generated between them. In such grafting experiments, mesenchyme and neural crest cells were included in the piece of donor tissue. Pieces of donor tissue about 1.75 rhombomeres in length, from 1 side of the hindbrain, were brought into apposition with host tissue, having removed all boundary cells at the site of interest (Fig. 1). One side was left undisturbed as a control. Several categories of grafting experiment were used; rhombomeres from adjacent positions, rhombomeres from positions three segments distant from one another, rhombomeres from the same position, odd-numbered combinations, and even-numbered combinations. Two permutations were used in each category. Following the transplantation, embryos were grown up for 24–48 h and then analysed either as flat-mounted brains to examine the pattern of boundaries, or processed for immunohistochemistry using antibodies against a neurofilament-associated protein.

The results of this study showed that, with one exception, each category of rhombomere combination

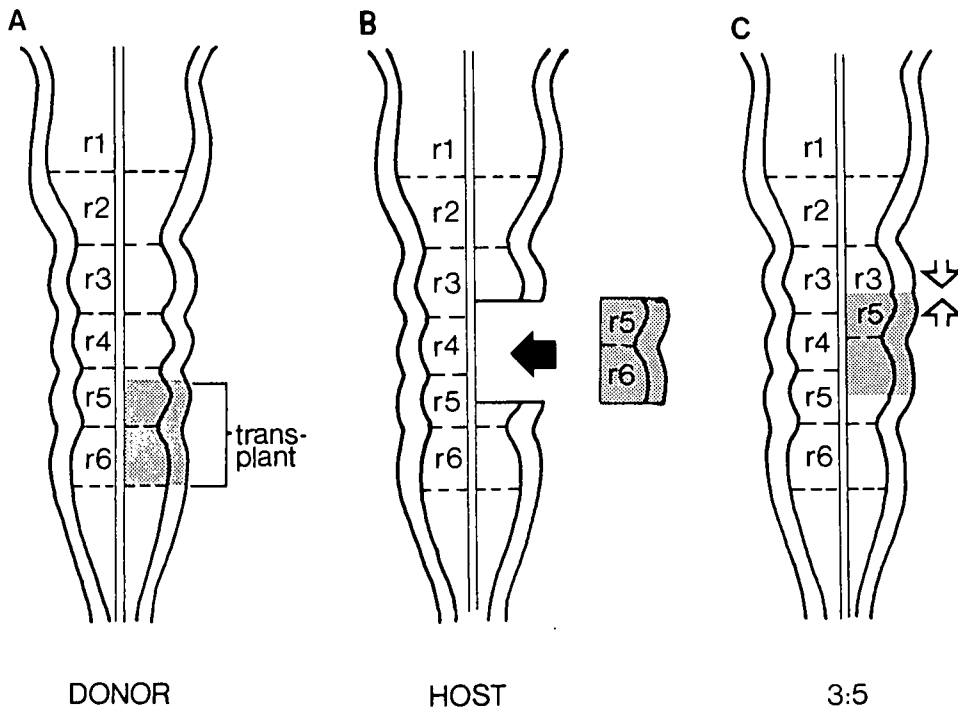


Fig. 1. Diagram of rhombomere transplantation experiments: example of juxtaposition of r3 with r5. (A) Stage 10–11 hindbrain of donor embryo (all rhombomere boundaries drawn as dotted lines, irrespective of whether they are morphologically apparent at this stage). Shaded area of r5 and r6 is removed, omitting the anterior quarter of r5. (B) Hindbrain of an isochronic host embryo, from which r4 and part of r5, plus the posterior quarter of r3, has been removed. The donor piece is then inserted, with correct polarity. (C) Host embryo once grafted piece is in place, showing that now r3 and r5 tissue is brought into apposition in the absence of pre-existing boundary cells.

yielded either a new boundary, or an oversized rhombomere with no internal boundary (Fig. 2). The confrontation of adjacent segments (r3:4 and r4:5) provided a parallel to the boundary ablation experiment, and yielded the expected result that a new boundary was produced in all cases. This was also true of experiments in which rhombomeres normally lying three segments distant from one another (r3:6 and r2:5) were juxtaposed. In contrast, segments of the same positional identity (r4:4 and r5:5) combined to give an enlarged rhombomere with no boundary. Combinations of the odd-numbered segments 3 and 5 also

gave the unequivocal result that a boundary was never formed. In grafts of even-numbered segments (r4:6 and r2:4), failure to form a boundary was also the majority result, though in some cases ($n=4/19$) there was evidence of a partial or an entire boundary. Surprisingly, the combination of odd-numbered segments r5 and r7 did not conform to this rule, giving rise to a new boundary in all cases.

Based on these data, it appears that the territory of rhombomeres 2 to 6, bounded by the r1/2 and r6/7 boundaries, form a pattern of alternating cell surface properties. Rhombomeres 2, 4 and 6 share similar

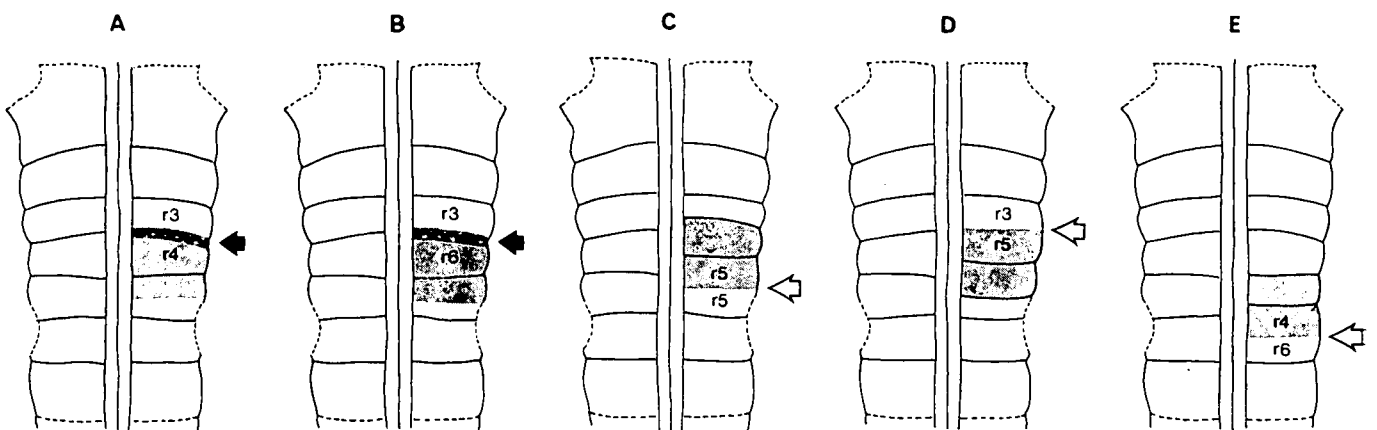


Fig. 2. Summary diagrams showing hindbrain morphology following rhombomere grafting experiments. In each case, shading denotes the grafted piece. In (A) and (B), heavy shading and black arrow show the formation of a new rhombomere boundary. In (C), (D) and (E), white arrow shows the junction between the host and graft tissue, within an enlarged rhombomere. (A) Results of r3:4 (also r4:5) grafts, showing generation of a new boundary. (B) Results of r3:6 (also r2:5) grafts, showing generation of a new boundary. (C) Results of r5:5 (also r4:4) grafts, showing formation of an enlarged rhombomere with no boundary. (D) Results of r3:5 grafts, showing formation of an enlarged rhombomere with no boundary. (N.B. r5:7 grafts gave a different result, always generating a new boundary). (E) Results of r4:6 (also r2:4) grafts, which in a majority of cases gave rise to an enlarged rhombomere with no boundary.

properties, as do rhombomeres 3 and 5. Whether these differences are on the level of single molecules is not yet known. It may be more probable that individual rhombomeres have distinct cell surface molecules, but that those of r3 more closely match those of r5 than those of r2, r4 and r6. Rhombomere 7 does not appear to fit into this schema. Within the hindbrain region, the segmental quality seems to fade out caudally, the rhombomere boundaries r7/8 and r6/7 being less morphologically clear than those further rostrally. In addition, neurogenesis in r7 is not delayed in the same way as in r3 and r5. Thus, r7 may exhibit properties intermediate between those of the hindbrain and the spinal cord.

The phenomenon of rhombomere boundary regeneration bears a resemblance to segment boundary regeneration in the insect *Oncopeltus*. Here, confrontation of adjacent segmental tissue in the absence of boundary cells also led to boundary regeneration (Wright and Lawrence, 1981a). When cells of different colour were confronted in this way, the boundary was observed to reform, first along an irregular interface that was later refined to a straight line (Wright and Lawrence, 1981b). This suggested to the authors that there were adhesive differences between the cells of adjacent segments. At present, we are engaged in repeating these rhombomere grafting experiments using quail-chick chimaeras, to examine the prevalence of cell mixing in paradigms where either a new boundary is formed, or a boundary is not regenerated. If the immiscibility of cells in adjacent territories is the mechanism used in the generation of cell lineage restrictions and the basis of the rhombomeric pattern, then we would expect, for example, that in a combination of r3:r5, cells would mix freely with each other, whereas in cases of boundary regeneration, eg. r4:r5, cells would segregate along the regenerating boundary interface. One finding supporting the idea of a periodicity in cell surface properties in the hindbrain is the observed prevalence of glycoproteins carrying the HNK-1 epitope in rhombomeres 3 and 5 (Kuratani, 1991).

Segmentation of the neural crest

In addition to the intrinsic segmentation of the hindbrain, there is evidence to suggest that the hindbrain-derived neural crest plays a role in patterning the head region. Mesodermal derivatives such as the myoblasts of the branchiomeric muscles are patterned by the neural crest (Noden, 1986), and crest cells from the first branchial arch level appear to be morphogenetically specified before they migrate, since first arch crest grafted into second arch position will give rise to elements of supernumerary first arch structures (Noden, 1983).

In the chick embryo, scanning electron micrographs showed neural crest cells migrating into the branchial arches as cords of cells (Anderson and Meier, 1981). So far, the patterns of crest migration in the head region

have not been mapped at a fine level. Previous studies employing orthotopic grafts of tritiated thymidine-labelled crest cells into unlabelled hosts, mapped neural crest cell migration from several cranial levels, including two that encompassed the hindbrain; the metencephalon and the rostral myelencephalon (Noden, 1973, 1975). Crest cells from the metencephalon (and the mesencephalon) were found to populate the trigeminal ganglion and the mandibular arch while those from the myelencephalon populated the geniculate ganglion and the hyoid arch. Another study, which used the quail-chick chimaera technique, agreed that mesencephalic crest colonised the mandibular arch, and also the trigeminal ganglion, maxillary process and developing eye (Le Lievre and Le Douarin, 1975). However, cells from the rhombencephalon were found mainly to colonise the second, third and fourth arches, and were only rarely found at trigeminal-mandibular arch levels.

In order to examine in detail the patterns of crest migration, focal injections of the highly fluorescent dye, DiI (Honig and Hume, 1986) were made into the dorsal midline neural folds of chick embryos to label the premigratory neural crest (Lumsden *et al.* 1991). Injections were small and restricted in the anteroposterior axis in order to examine the specific axial levels of crest cell migration. This analysis was confined to posterior mesencephalic and rhombencephalic levels. After labelling, embryos were then grown up for 24–48 h, and analysed using intensified video microscopy to examine the distribution of labelled crest cells. In particular, the question addressed was whether crest cells from specific axial levels populated particular branchial arches.

In DiI-injected embryos, the labelled cells included the neural crest cells that had migrated away from the injection site, while the dye that remained in the hindbrain allowed retrospective visualisation of the location and extent of the DiI injection. In the chick, migration of the mesencephalic crest occurs between stages 9– and 10+, while that of the rhombencephalic crest occurs between stages 9+ and 11 (Tosney, 1982). Injections spanned this time interval from stage 8– to stage 11.

Spatial distribution of crest cells

Early injections (stage 8– to 8) at midbrain levels or into the posterior midbrain-rhombomere 1 resulted in labelled cells being distributed dorsally to the developing eye, in the maxillary process and the rostral half of the mandibular process. Embryos injected later contained labelled cells in the trigeminal ganglion, the entire mandibular arch and the maxillary process, though by stage 9+ there was no labelling of the maxillary process. While earlier injections tended to show some cells in the ophthalmic branch of the trigeminal ganglion, later ones were confined to the maxillomandibular division, and in addition, only a few cells were seen in the caudal half of the mandibular process.

For injections into rhombomere 1/rhombomere 2 (r1/r2) at stage 8–, crest cells were located in the trigeminal ganglion and the caudal half of the mandibular arch. At stage 8 and 8+ the trigeminal ganglion and the whole of the mandibular arch were labelled, but from stage 9+ onwards, only the caudal half of the mandibular process and the maxillomandibular lobe of the trigeminal ganglion were colonised by crest cells. Injections into rhombomere 2 gave rise to crest cells in only the mandibular lobe of the trigeminal ganglion and the caudal half of the mandibular process.

At r2/r3 levels, injections at stages 8– and 9– filled the trigeminal ganglion and the mandibular and maxillary processes. By stage 9– and 9+, labelled cells were found only in the mandibular lobe of the trigeminal ganglion and the caudal half of the mandibular process. By stage 11, the degree of labelling had decreased in the mandibular arch, and was predominant within the mandibular lobe of the trigeminal ganglion. After injections made exclusively into rhombomere 3, labelled crest cells were not observed outside the neuroepithelium, suggesting that labelling resulting from r2/r3 injections was due to emigration of crest cells from r2 only. This idea is reinforced by the finding that injections into the r3/r4 region yielded the same result as those made into r4 alone, that is, labelling of the geniculate and vestibuloacoustic ganglion, and of the hyoid arch. In a similar fashion to injections into r3, those into r5 did not result in the labelling of any migratory crest cells. Injections into the r4/r5 region thus gave the same result as those into r4 alone. When injections spanned the r4/5/6 region, labelled crest cells entered both the second and third branchial arches, after migrating rostrally and caudally of the otocyst respectively. Crest cells labelled in r6 were later located in the superior and petrosal ganglia of the glossopharyngeal nerve, and the third branchial arch. r6/r7 injections gave the result that labelled cells were located in the third and fourth branchial arches and in the petrosal ganglion.

Neural crest cells are thus divided into groups at discrete segmental levels, which migrate as segregated streams (summarised in Fig. 3). Crest cells originating from the level of r1/r2 populate the trigeminal ganglion and the mandibular arch, and to a lesser extent the maxillary process. Crest cells from the level of rhombomere 4 populate the geniculate–vestibular ganglion and the hyoid arch. The group of cells originating from rhombomere 6 populates the superior and petrosal ganglion and the third branchial arch. The intervening regions of rhombomeres 3 and 5 do not produce migratory neural crest.

The time course with which crest cells filled various structures down the rostrocaudal axis indicated a rostrocaudal wave of maturation of neural crest cells in the hindbrain region. For example, early injections into the r1–r3 region tended to result in filling of the whole trigeminal ganglion, while those at later time points resulted in filling of only the maxillomandibular lobe. This could either represent a progressive change in fate of the cells of a particular region, or a more passive

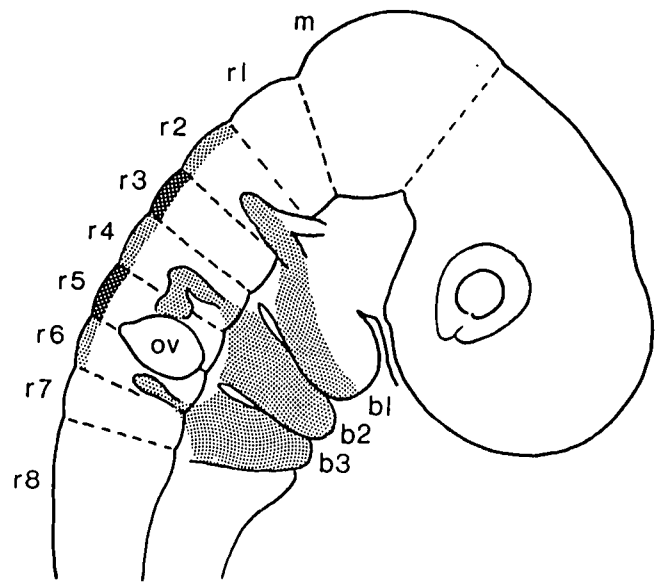


Fig. 3. Summary diagram showing neural crest cell migration paths from rhombomeres r2–r6. The branchial arches (b1–b3) are populated by cells from the even-numbered rhombomeres 2, 4 and 6 (light stipple) but not from r3 or r5 (heavy stipple).

rostrocaudal filling of available space. In addition, there was a ventral to dorsal progression in the population of structures at successively later time points. For injections into the r2–r3 region this meant that cells labelled early were more likely to contribute to mandibular arch derivatives while those labelled late had an increased tendency to contribute to the trigeminal ganglion.

Segmentation of the head

In their search for a unifying ontogenetic and phylogenetic theory of head segmentation, the early comparative anatomists focused on the disposition of the mesoderm (Goodrich, 1930), with the idea that the head was a modification of the trunk. In the trunk the mesoderm is conspicuously divided into somites, and though far less conspicuous in the head, segmentation of the paraxial mesoderm was nonetheless seen as the driving force of segmentation in that region also. More recent studies, however, have pointed out that developmental strategies in the head differ from those in the trunk in a number of fundamental ways (Gans and Northcutt, 1983). In vertebrates the head region is not dominated by segmental muscles which produce bending movements, except for the branchial region; instead muscles are designated for individual specialised functions, and are supplied by specific cranial nerves.

The cranial neural crest had a considerable role in bringing about these specialised functions of the vertebrate head. In addition to giving rise to the sensory and autonomic ganglia, as it does in the trunk, it also forms tissues from cells produced elsewhere by the mesoderm (Le Lievre and Le Douarin, 1975; Le Lievre, 1978; Noden, 1978, 1983). The cranial neural crest also

has a role in patterning mesodermal derivatives (Noden, 1986). It has been suggested that the neural crest is positionally specified before migration, since presumptive first arch crest grafted ectopically can give rise to supernumerary first arch structures (Noden, 1983). Invading myogenic and angiogenic populations may all receive spatial cues from the neural-crest derived connective tissue (Noden, 1988). The paraxial mesoderm thus appears to be a passive element in the patterning process, reducing its potential significance in head segmentation. Nevertheless, some authors have proposed that the head neural crest migration might be guided, or at least correlated, with the presence of cryptic mesodermal segments. In some embryos, notably newt and turtle, the mesoderm appears to be quasi-segmented into somitomeres (Jacobson and Meier, 1984; Meier and Packard, 1984), but the status of these structures is controversial (Meier, 1981; Jacob *et al.* 1986).

In the trunk, the migration pathways of neural crest cells are indeed determined by the adjacent mesoderm. Crest cells emigrate from the neural tube all the way down the axis, but move only through the rostral and not the caudal halves of the adjacent sclerotome (Rickmann *et al.* 1985). While rostral half-somites provide an environment permissive for crest migration and axon outgrowth, caudal half-somites do not (Stern *et al.* 1986; Davies *et al.* 1990). Rostral and caudal half sclerotomes exhibit molecular differences, in for example the distribution of fibronectin, tenascin and peanut-agglutinin-binding proteins (Erickson, 1986; Mackie *et al.* 1988; Stern *et al.* 1986). None of these differences are present in the mesoderm of the head region, however, implying instead that the pre-migratory crest might itself be segmented. The surface ectoderm may help guide crest migration (Lofberg *et al.* 1985), in addition to other local cues.

Conclusions

Since the neural crest organises the mesoderm of the branchial arches, segmentation of the neural crest would establish a dynamic association between segmentation in the brain and the branchial arches. A specific cranial nerve, for example, would innervate a branchial arch muscle with a shared rhombomeric level of origin. Whether this matching mechanism is instrumental in setting up correct innervation patterns in the branchial region, or merely incidental to it, is not yet known. Within the neural tube, a two-segment periodicity in cell surface properties would be matched to the two-segment periodicity of neural crest production. The ectoderm may also be divided into segmental units or 'ectomeres' in register with the rhombomeric arrangement (Couly and Le Douarin, 1990).

The time at which segmental identity is specified in the hindbrain region is not known. Differential adhesive properties between rhombomeres may be established before the morphological appearance of the first boundaries at stage 9-. The juxtaposition of

domains with different cell surface molecules may then trigger the generation of boundary properties at the interface, such the expression of low-PSA N-CAM and a reduced rate of cell division. At this interface, other differentiated properties of the boundary are then progressively established. The segmentation in the neural crest must occur prior to stage 9+, when the first rhombencephalic crest cells emerge (Tosney, 1982).

The specification of alternating patterns in the hindbrain may be linked to genes of the *Hox 2* cluster, which are expressed in the neural crest (Hunt *et al.* 1991), and in the hindbrain neuroepithelium, in domains that terminate at rhombomere boundaries (Wilkinson *et al.* 1989b). Groups of crest cells that populate specific branchial arches express different subsets of *Hox 2* genes. In addition, the zinc-finger gene *Krox 20* is expressed in a segment-restricted manner, in rhombomeres 3 and 5 (Wilkinson *et al.* 1989a), and in the cranial ganglia adjacent to the even-numbered segments. Future work will focus on the molecular basis of the alternating periodicity of cell surface properties in the hindbrain, and its relationship to known, and as yet uncharacterised genes.

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